High-resolution subtyping of Staphylococcus aureus strains by means of Fourier-transform infrared spectroscopy

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Abstract: Staphylococcus aureus causes a variety of serious illnesses in humans and animals. Subtyping of S. aureus isolates plays a crucial role in epidemiological investigations. Metabolic fingerprinting by Fourier-transform infrared (FTIR) spectroscopy is commonly used to identify microbes at species as well as subspecies level. In this study, we aimed to assess the suitability of FTIR spectroscopy as a tool for S. aureus subtyping. To this end, we compared the subtyping performance of FTIR spectroscopy to other subtyping methods such as pulsed field gel electrophoresis (PFGE) and spa typing in a blinded experimental setup and investigated the ability of FTIR spectroscopy for identifying S. aureus clonal complexes (CC). A total of 70 S. aureus strains from human, animal, and food sources were selected, for which clonal complexes and a unique virulence and resistance gene pattern had been determined by DNA microarray analysis. FTIR spectral analysis resulted in high discriminatory power similar as obtained by spa typing and PFGE. High directional concordance was found between FTIR spectroscopy based subtypes and capsular polysaccharide expression detected by FTIR spectroscopy and the cap specific locus, reflecting strain specific expression of capsular polysaccharides and/or other surface glycopolymers, such as wall teichoic acid, peptidoglycane, and lipoteichoic acid. Supervised chemometrics showed only limited possibilities for differentiation of S. aureus CC by FTIR spectroscopy with the exception of CC45 and CC705. In conclusion, FTIR spectroscopy represents a valuable tool for S. aureus subtyping, which complements current molecular and proteomic strain typing.

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1 ***S. AUREUS* SUBTYPING BY FTIR SPECTROSCOPY**

2 **High-resolution subtyping of *Staphylococcus aureus* strains by means of Fourier-**
3 **transform infrared spectroscopy**

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ABSTRACT (max.250)

Staphylococcus aureus causes a variety of serious illnesses in humans and animals. Subtyping of *S. aureus* isolates plays a crucial role in epidemiological investigations. Metabolic fingerprinting by Fourier-transform infrared (FTIR) spectroscopy is commonly used to identify microbes at species as well as subspecies level. In this study, we aimed to assess the suitability of FTIR spectroscopy as a tool for *S. aureus* subtyping. To this end, we compared the subtyping performance of FTIR spectroscopy to other subtyping methods such as pulsed field gel electrophoresis (PFGE) and *spa* typing in a blinded experimental setup and investigated the ability of FTIR spectroscopy for identifying *S. aureus* clonal complexes (CC). A total of 70 *S. aureus* strains from human, animal, and food sources were selected, for which clonal complexes and a unique virulence and resistance gene pattern had been determined by DNA microarray analysis. FTIR spectral analysis resulted in high discriminatory power similar as obtained by *spa* typing and PFGE. High directional concordance was found between FTIR spectroscopy based subtypes and capsular polysaccharide expression detected by FTIR spectroscopy and the *cap* specific locus, reflecting strain specific expression of capsular polysaccharides and/ or other surface glycopolymers, such as wall teichoic acid, peptidoglycane, and lipoteichoic acid. Supervised chemometrics showed only limited possibilities for differentiation of *S. aureus* CC by FTIR spectroscopy with the exception of CC30, CC45, and CC705. In conclusion, FTIR spectroscopy represents a valuable tool for *S. aureus* subtyping, which complements current molecular and proteomic strain typing.

Key words: *Staphylococcus aureus;* subspecies typing; FTIR spectroscopy; capsular polysaccharide; surface glycopolymer; DNA microarray

INTRODUCTION

Subtyping of *Staphylococcus aureus*, an organism causing a wide range of life-threatening infections and toxinoses, is crucial to epidemiological investigations and phylogenetic studies [5,23]. Common techniques used for subtyping of *S. aureus* are pulsed field gel electrophoresis (PFGE), *spa* typing, and multilocus sequence typing (MLST) [1,11,34]. However, PFGE exhibits grave performance discrepancies when used in inter-laboratory comparisons [45], *spa* typing may fail to recognize new lineages as a result of inherent homoplasy and differing evolutionary rates of *spa* alleles [33], and MLST has been shown to offer only moderate discriminatory power for epidemiological studies at comparatively high cost [15,16]. In addition, these techniques rely on molecular genotyping and do not allow insights into phenotypic features.

In contrast, Fourier-transform infrared spectroscopy (FTIR) represents a technique that enables not only bacterial identification, but also phenotypic characterization. FTIR spectroscopy measures the overall chemical composition of a sample, thus creating a spectrum that can be used as a fingerprint and that is analyzed by pattern recognition algorithms [42]. It is a promising tool used for microbial identification and was shown to yield resolution power sufficient for typing of bacteria and yeast below the species level [14,28,43]. For instance, FTIR was used for identification of methicillin-resistant *S. aureus* [2], capsular serotypes [18], the examination of small-colony variants [4], and for the rapid discrimination of strains involved in an outbreak investigation [23].

In the presented study, the *S. aureus* subtyping performance of FTIR spectroscopy, PFGE, and *spa* typing was compared using discriminatory power (Simpson's index of diversity) and directional congruence (adjusted Wallace coefficient) [28] [7] as performance criteria.

MATERIAL AND METHODS

Bacterial strains. The 70 *S. aureus* strains used in this study originate from a collection of well-characterized strains isolated from human, animal, and food sources by the Institute for Food Safety and Hygiene, University of Zurich. DNA microarray profiles, including assignment to clonal complexes (CC) and virulence and resistance gene patterns of all strains, were generated in previous studies [13,22,25,38]. All 70 strains showed unique DNA microarray hybridization patterns. The strain set comprises 10 strains each of CC5, CC8, CC30, CC45, CC398, and CC705 (former CC151), as well as two strains each of CC9, CC12, CC15, CC22, and CC97. Clonal complexes were selected to represent *S. aureus* commonly isolated from asymptomatic and infected humans (CC5, CC8, CC30, and CC45) and animals (CC705, CC398). Strains of CC705 cause severe losses to the dairy industry due to mastitis in cattle and strains assigned to CC398 have been linked to livestock associated MRSA infections in humans. To be able to test the subtyping performance of the different methods for a more diverse strain set, two strains each of five additional clonal complexes (CC9, CC12, CC15, CC22, CC97) were included. Strains belonging to CC45 and CC705 exhibited the highest degree of homogeneity regarding virulence and resistance gene profiles determined by DNA microarray. Other clonal complexes such as CC5, CC8, CC22, and CC398 exhibited a higher degree of variation with regard to the presence/absence of virulence and resistance genes. A comprehensive overview of strain sources, clonal complexes, as well as virulence and resistance gene profiles is provided as a supplemental file (Supplement 1). The diversity of the strain set is illustrated by a SplitsTree calculated as previously described [39] from DNA microarray hybridization profiles (Supplement 2). To assure unbiased processing, strains were assigned random numbers before being blinded analyzed by FTIR spectroscopy. *S. aureus* Reynolds prototype strain CP5 and its isogenic mutants Reynolds CP8 and Reynolds CP(-) (nonencapsulated) were used as controls for genotyping as well as for FTIR spectroscopic biotyping [40].

FTIR spectroscopic measurement and spectral preprocessing. FTIR spectroscopic measurements, spectral quality determination, spectral preprocessing, and chemometric analysis were performed as previously reported [18,24]. Three independent experiments were performed on different days. Isolates were grown as a bacterial lawn on tryptone soy agar plates (Oxoid) for 24 h at 30°C and measured by FTIR spectroscopy. One loopful of bacterial cells was suspended in 100 µl sterile deionized water. An aliquot of 30 µl of the suspension was spotted on a zinc selenite (ZnSe) optical plate and dried at 40°C for 40 min to yield transparent films, and subsequently submitted to FTIR spectroscopic measurement. FTIR spectra were recorded in transmission mode in the spectral range of 4,000-500 cm^{-1} with an HTS-XT microplate adapter coupled to a Tensor 27 FTIR spectrometer (Bruker Optics GmbH, Ettlingen, Germany). OPUS software (Version 6.5; Bruker Optics GmbH) was used for FTIR spectroscopic data analysis. Original spectra were preprocessed: (1) second derivatives were calculated over the whole spectral range using a second-order 9-point Savitzky-Golay algorithm, (2) spectra were vector normalized. In order to obtain the levels of reproducibility among the replicate samples, the averages plus/minus 2 standard deviations (SD) of the D-value, which is based on the Pearson's product moment correlation coefficient [8,21,30] were calculated in the spectral range used for typing (1200-800cm^{-1}). Assuming that by strictly following the sample protocol a D-value below 0.50 is achievable, spectra with sufficient reproducibility can be obtained [41,43].

Unsupervised and supervised chemometrics.

For hierarchical cluster analysis (HCA), the spectral region that offers the maximum information and discriminatory power (1,200-800 cm^{-1}) was selected, which is dominated by C-O-C and C-O-P stretching vibrations of various oligosaccharides and polysaccharides and their specific types of glycosidic linkages. The dendrogram was generated using Ward's algorithm at repro-level 30. Strains were considered to be distinguishable, if one or more strain(s) formed a subcluster that comprised all three repetitive measurements and was clearly

129 separated from subclusters of other strains [41]. Capsular serotypes (CP5, CP8, NT) were
130 determined using artificial neuronal network (ANN) analysis [18]. The determination of
131 capsule types included *S. aureus* strain Reynolds CP5 and its isogenic mutants Reynolds CP8
132 and Reynolds CP- as control strains for CP expression [40]. To investigate the ability of FTIR
133 spectroscopy for identifying *S. aureus* clonal complexes, unsupervised principal component
134 analysis (PCA), supervised principal component and linear discriminant analysis (PCA-
135 LDA), and support vector–machine classification (SVMC) was performed using the software
136 Unscrambler X (CAMO Software, Oslo, Norway) applying the same spectral windows as
137 mentioned above. PCA computation was based on the NIPALS algorithm and three
138 components were projected for PCA-LDA. SVMC used a radial basis function as Kernel type
139 and the optimal C-value and Gamma was predetermined using the grid search function. For
140 PCA-LDA and SVMC, 70% of randomly selected isolates of each CC were used to set up the
141 model and 30% for external validation.
142 ***cap* gene determination.** The presence of the respective *cap* genes defining the CP5 and CP8
143 serotypes was determined by PCR amplification using primers and conditions described
144 elsewhere [19,37].
145 ***spa* typing.** The polymorphic X region of *spa* was determined as previously described [39].
146 Briefly, *spa* was amplified using the following primers: spa-1113f (5'-TAA AGA CGA TCC
147 TTC GGT GAG C-3') and spa-1514r (5'-CAG CAG TAG TGC CGT TTG CTT-3') [1]. The
148 GoTaq PCR system (Promega AG, Dübendorf, Switzerland) was used at the following
149 reaction conditions: (i) 5 min at 94°C; (ii) 35 cycles of 45 s at 94°C, 45 s at 60°C and 90s at
150 72°C; and (iii) 10 min at 72°C. Amplicons were purified (MinElute PCR Purification Kit,
151 Qiagen, Hilden, Germany) and sequencing was outsourced (Microsynth, Balgach,
152 Switzerland). The *spa*-server (http://spa.ridom.de/) was used to assign nucleotide sequences to
153 *spa* types [20].

PFGE analysis. Chromosomal DNA was prepared and PFGE analysis of *Sma*I digested fragments was performed as described elsewhere [3]. Electrophoresis was carried out in a Bio-Rad CHEF-DR III electrophoresis cell and *Salmonella enterica* serovar Braenderup strain H9812 digested with 50 U *Xba*I for 12 h at 37°C was used as a molecular size standard. Gels were analyzed with Gel Compar II software (Applied Maths, Sint-Martens-Latem, Belgium) using the dice coefficient and were represented by unweighted pair grouping by mathematical averaging (UPGMA) with an optimization of 0.5%, and position tolerance of 1%. Different patterns were grouped into pulso-types based on a cutoff value of 95% similarity.

Analysis of strain typing results. Discriminatory power, typability and typing concordance was assessed using the publicly available online plattform Comparing Partitions (http://darwin.phyloviz.net/ComparingPartitions/index.php?link=Tool). Discriminatory power was determined by calculating the Simpson's index of diversity (SID), which quantifies the probability of each typing method to discriminate between unrelated strains [32]. In order to avoid overestimating the discriminatory power of the typing system, all non-typable strains were assembled into a single group. However, this does not imply that they are of the same type [5]. SID should be at least in order of 0.95 for a typing system to be considered ideal [5], and SID > 0.95 was suggested for outbreak investigations if the typing results are to be interpreted with confidence [35]. The confidence intervals (95%) of SID values were calculated as published previously [17]. The quantitative concordance between typing methods and strain characteristics were calculated by using the adjusted Wallace's coefficient [10,31]. The adjusted Wallace's (AW) coefficient represents the probability that a pair of strains that have the same type in method/ characteristic 1 share the same type in method/ characeristic 2. An AW coefficient close to 1.0 indicates high congruence of results between methods. In contrast to the Wallace's coefficient, it provides the overall concordance taking into account that the agreement between the typing methods is not due to chance alone.

RESULTS

Subtyping by FTIR spectroscopy. FTIR spectroscopy coupled to HCA was conducted to assess overall similarity between isolates of different *S. aureus* lineages (Fig. 1). Strains were put in randomized order and were measured and analyzed blinded. Subtyping by FTIR spectroscopy was highly discriminatory as calculated by the Simpsons index of similarity (SID = 0.983) (Table 1). Among the 70 isolates, 49 subtypes could be distinguished and were divided into distinct subclusters. Each subcluster indicates the co-alignment of triplicate spectra from a single strain or a group of not further distinguishable strains. Replicate measurements of two strains (strains 17 and 28) could not be assigned to a single subcluster (classified as non-typable), but were allocated in proximate subclusters. The subtype with the highest number of isolates was a cluster comprising seven CC45 strains of different *spa* types (Fig. 1; main cluster of A1).

Comparison of the subtyping performance of FTIR spectroscopy, PFGE, and *spa* typing. A comprehensive overview of the typing performance parameters of all tested techniques is given in Table 1. FTIR subtyping (SID = 0.983), PFGE (SID = 0.979) and *spa* typing (SID = 0.973) revealed similar SID values, and therefore similar discriminatory power at a 95% confidence level. However, PFGE also exhibited the highest number of non-typable strains, as ten out of 70 isolates could not be typed due to failed *SmaI* restriction. The ten strains exclusively belonged to CC398. In contrast, *spa* typing yielded results for all isolates, but did not allow for differentiation of the closely related CC705 strains (all t529), which were discriminated by FTIR spectroscopy into six subtypes (Fig. 1, cluster A2). The AW coefficient for the quantitative measures of congruence between typing methods was below 0.195 when examined in both directions (Table 2). Hence, there was only a very low probability that isolates assigned to the same cluster by *spa* typing and PFGE would be assigned to the same cluster by FTIR subtyping and vice versa. Comparison of the more general strain characteristics indicated a strong correlation of the *spa* type in the direction of

CC (0.929), and *agr* type (0.926). Thus, the probability of two strains having the same *spa* type sharing the same CC and *agr* type is >90% (Table 2).

Insufficient discrimination of *S. aureus* clonal complexes by FTIR spectroscopy

Sixty *S. aureus* isolates representing six major clonal complexes (CC5, CC8, CC30, CC45, CC398, and CC705; 10 isolates each) were included in the analyses. Unsupervised PCA of FTIR spectra showed only limited possibilities for differentiation of *S. aureus* CC by FTIR spectroscopy. This is visualized in Fig. 2, illustrating that clusters of isolates assigned to the same CC exhibited various degrees of overlap. Only isolates belonging to CC45 and CC705 were mostly grouped in distinct coherent clusters.

Supervised classification of FTIR spectral data from the same data set was conducted by means of PCA-LDA and SVMC. Classification accuracy for PCA-LDA and SVMC equaled 66.7% and 100% for the training set, and in total 77.8% and 83.3% for external validation, respectively. Apart from these rather moderate classification outcomes of the external validation, a 100% correct classification was realized for strains, which belong to CC8, CC45, and CC705 for PCA-LDA and CC30, CC45, and CC705 for SVMC. Neither changing the spectral pre-processing nor the spectral ranges resulted in improved classification results.

FTIR spectroscopic subtyping is primarily based on bacterial glycopolymers. Grouping based on CP expression using ANN-assisted FTIR spectroscopy showed that 28.6% (n = 20) expressed CP5, 30.0% (n = 21) expressed CP8, 38.6% (n = 27) were characterized as non-typable (NT), and 2.8% (n = 2) were classified as highly variable for CP expression. Clustering results reflected CP serotypes, with strains being divided into three main clusters A, B, and C, which almost exclusively comprised strains assigned to CP8, NT, and CP5, respectively (Fig. 1). The strain specific assignments of the respective *cap* alleles, *cap1*, *cap5* and *cap8* were shown in Fig. 1 and accounted for 0%, 47% (n=33) and 53% (n=37), respectively.

DISCUSSION

Our strain set represented a diverse group of *S. aureus* isolates which showed unique DNA microarray hybridization patterns and were collected from different human, animal, and food sources comprising the most dominant CC. For many laboratories, PFGE is still the method of choice for DNA fingerprinting of *S. aureus,* with *Sma*I digestion being considered the gold standard [26]. The discriminatory power obtained for PFGE was 0.979, which is comparable to findings of previous studies [11,12]. However, in our study, all strains assigned to CC398 were non-typable using this method. This is consistent with previous reports of ST398 isolates being refractory to *Sma*I restriction based on methylation [6]. This is of particular relevance in view of the emergence of livestock-associated methicillin-resistant *S. aureus* (MRSA) of ST398.

In the current study, the use of FTIR spectroscopy followed by HCA for subspecies-level typing of *S. aureus* resulted in high discriminatory power (SID = 0.983), comparable to *spa* typing (SID = 0.973) and PFGE (SID = 0.979). In contrast to PFGE and *spa* typing, FTIR spectroscopy yields much faster results and is also suited for high-throughput applications. Since FTIR subtyping may discriminate between strains found to be clonal by PFGE and may provide additional information such as data on CP expression, a combination of these two typing methods would create added value. FTIR spectroscopy could be particularly useful for differentiating strains in the context of source attribution studies, transmission route analyses and outbreak investigations [20].

The AW coefficient allows the evaluation of the strength and directionality of the correspondences between the methods/ characteristics. A high AW coefficient indicates that results obtained by a given method/ characteristic could have been predicted from the results of another method, suggesting that the use of both methods is redundant [7]. High directional concordance was found between CP expression and the *cap* specific locus (AW coefficient $_{\text{FTIR}\rightarrow\text{CP type}} = 0.965$; $_{\text{FTIR}\rightarrow\text{cap locus}} = 0.953$, respectively) reflecting strain specific expression of

CPs or other surface-associated glycostructures, rather than assignment clonal complexes (AW coefficient $_{FTIR \rightarrow CC}$ = 0.758, respectively). Further, the AW coefficient revealed strong asymmetries depending on the directionality, which suggests that the results did not rely on a particular clonal composition of the studied strain collection but are general properties of the typing methods used [10]. This was supported by unsupervised (PCA) and supervised (PCA-LDA, SVMC) chemometric analysis, which was not sufficiently discriminative to allow a consistent and reliable identification of clonal lineages, with the exception of CC45 and CC705. This partial correct matching of FTIR biotypes and CC is likely due to the fact that the capsule genotype correlates closely with the MLST clonal complex: CC5, *cap5*; CC8, *cap5*; CC30, *cap8*; CC45, *cap8*; CC705, *cap8* and CC398, *cap5* [27].

ANN-assisted FTIR spectroscopy enabled rapid and reliable CP typing, thus allowing for differentiation between *S. aureus* serotypes CP5, CP8, and NT [18]. Analysis of the normalized 2nd derivative spectra in the 1,200-800 cm^{-1} region showed spectral differences particularly at 834 cm^{-1}, which might be related to specific structural α- and β-glycosidic linkages between *S. aureus* CP5 and CP8 strains [18]. High-resolution separation was even achieved for *S. aureus* NT strains (27 NT isolates/ 24 clusters), which were reported not to express a capsule. Thus, other surface glycopolymers, such as wall teichoic acid (WTA), peptidoglycane, and lipoteichoic acid must be causative for the precise differentiation of NT strains. The observed heterogeneity of *S. aureus* glycotypes supports the assumption that the differential expression of bacterial surface glycopolymers might play an important role in staphylococcal pathogenesis including surface recognition, cell adhesion or immune evasion [7,29]. The importance of surface glycopolymers in host-pathogen interaction, such as CP expression and WTA glycosylation, has recently been reported to be associated with *S. aureus* chronicity, colonization and resistance [9,36,44].

CONCLUSION

Our study revealed high discriminatory power of FTIR spectroscopy-based subtyping of *S. aureus* comparable to that of *spa* typing and PFGE. FTIR spectroscopic strain discrimination does not yield results fully congruent with those of *spa* typing and PFGE, as it is primarily based on the differential expression of capsular serotypes and/ or additional surface glycopolymers rather than assignment to clonal complexes. We were able to show that FTIR spectroscopy is of value for *S. aureus* subtyping and provides phenotypic data on CP types. Therefore, FTIR spectroscopy may be particularly useful in source attribution studies and outbreak investigations, and could also contribute to the better understanding of the role of surface glycopolymers in *S. aureus* pathogenicity.

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CONFLICTS OF INTEREST

None.

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FIGURE CAPTIONS & TABLES

Figure 1

FTIR spectroscopy-based dendrogram of the analyzed strain set. Each strain was measured three times independently and clustering is mainly determined by the capsule type. If replicate measurements could not be assigned to a single subcluster, the number of asterisks indicates the number of repeated measurements assigned to each subcluster (**). CC: clonal complex, NT: non-typable, CP: capsular polysaccharide, HV: highly variable. Origin: SFP = staphylococcal food poisoning, NCO = nasal colonization, INF = infection, BOM = bovine mastitis, POU = poultry carcass, PIC = pig carcass, RAC = rabbit carcass.

Figure 2

Scores plot for the first three principal components obtained from FTIR spectra of *S. aureus* isolates of six different CC comprising 10 isolates per CC measured in triplicates.

Table 1

Comparison of the discriminatory power of FTIR spectroscopy, PFGE, and *spa* typing. The number (n) of typable isolates, partitions, singletons, and strains of the major type is given based on the analysis of a collection of 70 *S. aureus* strains.

	n typable isolates	n partitions incl. NT[a]	n singletons	n strains of major type	SID[b] (95% CI[c])
FTIR	68	50	41	7	0.983 (0.970-0.995)
PFGE	60	56	51	3	0.979 (0.958-1.000)
spa typing	70	48	39	10	0.973 (0.953-0.994)

[a] non-typable strains, which all assembled into a single group.

[b] Simpson's index of diversity

[c] confidence interval

501 **Table 2**

502 Cross-classification concordance. The table provides the adjusted Wallace coefficient for
503 typing based on different methods/ characteristics.

	adjusted Wallace coefficient						
	Typing methods			Strain characteristics			
	FTIR biotype	PFGE	*spa* type	CC	CP type	*cap* gene	*agr* type (I, II, III)
Typing methods							
FTIR biotype		0.051	0.195	0.758	0.965	0.953	0.854
PFGE	0.042		0.174	0.978	0.711	0.961	0.723
spa type	0.110	0.138		0.929	0.793	0.846	0.926
Strain characteristics							
CC	0.104	0.164	0.197		0.490	0.749	0.840
CP type	0.036	0.032	0.045	0.133		0.465	0
cap gene	0.017	0.021	0.050	0.099	0.228		0.036
agr type (I, II, III)	0.024	0.027	0.044	0.190	0	0.062	

504

505 CP, Capsular polysaccharide expression

506 CC, Clonal complex